

AD\_\_\_\_\_

Award Number: W81XWH-08-1-0348

TITLE: Stable Intravenous Fluorohydrocarbon Emulsion with High Oxygen Capacitance Combined with Hyperbaric Oxygen for the Acute Salvage of Tissue Injury After TBI

PRINCIPAL INVESTIGATOR: John R. Sims

CONTRACTING ORGANIZATION: Massachusetts General Hospital  
Boston, MA 02114

REPORT DATE: December 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>					
1. REPORT DATE (DD-MM-YYYY) 01-12-2009		2. REPORT TYPE Final		3. DATES COVERED (From - To) 1 JUN 2008-30 NOV 2009	
4. TITLE AND SUBTITLE Stable Intravenous Fluorohydrocarbon Emulsion with High Oxygen Capacitance Combined with Hyperbaric Oxygen for the Acute Salvage of Tissue Injury After TBI				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0348	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) John Sims Yi Zheng				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  Massachusetts General Hospital  Boston, MA 02114				8. PERFORMING ORGANIZATION REPORT	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The presence of hypoxia after traumatic brain injury (TBI) portends a worse outcome for recovery. Thus an important treatment target is the restoration of the disrupted oxygen delivery and utilization. Perfluorooctyl bromide (PFOB) has an unsurpassed ability to carry oxygen. Hyperbaric oxygen (HBO) has the ability to drive higher amounts of oxygen into fluids. These modalities combined together have the theoretical ability to maintain adequate oxygen delivery to the brain after traumatic brain injury (TBI). In C57 mice after mild and medium level of controlled cortical impact (CCI), we treated mice with vehicle only, hyperbaric oxygen only, PFOB only, and PFOB combined with HBOT, and then examined contusion volume (tissue loss), motor function and memory. Real-time measurements of local tissue oxygen partial pressure (PO2) in mice brain cortex demonstrated that PO2 of the injury core dropped to near zero after CCI. Significant reduction of PO2 was also observed in penumbra in the first hour after CCI. For functional test, Rotarod test, wire-grip, or Morris Water Maze, PFOB or HBO did not improve function. Brain tissue loss in oxygen treatment groups showed modest reduction but no significant improvement. Our study suggests that PFOB and/or HBOT at these doses and timing of delivery are harmful at high doses and not beneficial at lower doses.					
15. SUBJECT TERMS Hyperbaric oxygen, Perfluorooctyl bromide, fluorohydrocarbon, ischemia, traumatic brain injury, behavior, controlled cortical impact					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
U	U	U	UU	17	

## Table of Contents

	<u>Page</u>
Introduction.....	5
Body.....	6
Key Research Accomplishments.....	17
Reportable Outcomes.....	17
Conclusion.....	17
References.....	17
Appendices.....	18

## Introduction

Blast injury is a polytrauma injury. However in polytrauma, brain or intracranial injury account for 50% of all deaths. Beyond prevention of the injury, very little can be done about the primary insult. Thus, the job of the corpsmen, EMT or physician is to reduce the secondary insult. Although there are diverse primary insults ranging from extraaxial and intraaxial hemorrhage to axonal shearing to cortical contusion and penetrating projectiles, they all have a common pathway of secondary injury, that of altered oxygen delivery and utilization. Thus secondary injury follows with necrosis, edema, further blood-brain-barrier breakdown, increased intracranial pressure, worsening oxygen delivery with concomitant cell death and a vicious self-perpetuating cycle. The presence of hypoxia or hypotension in polytrauma dramatically increases the risk of death from traumatic brain injury (TBI), largely due to its contribution of ischemia and worsened oxygen delivery, which hastens the self-perpetuating cycle. What makes this cycle of metabolic failure difficult to treat in TBI is the extremely narrow therapeutic window, which is perhaps shorter than that of the 3-hour time window of stroke. Thus, safe therapeutic modalities will need to be invented for rapid deployment in the battlefield by corpsmen.

The ultimate tissue treatment would enhance oxygen delivery and utilization to the edematous and injured tissue to prevent progression of the cycle of cell loss. Simply applying oxygen by face mask does very little to increase oxygen delivery due to the fact that hemoglobin is the primary carrier of oxygen at atmospheric pressure and is almost completely saturated. Furthermore, in polytrauma with loss of blood the oxygen carrying capacity of blood will decrease with hemorrhage. Thus an easy to carry, safe, stable blood substitute with high oxygen carrying capacity is needed for the battlefield. Such compounds have been developed. These fluorohydrocarbons compounds, e.g., perfluorooctyl bromide (PFOB), have >20 times the ability to carry oxygen compared to blood. PFOB has the highest capacity to absorb oxygen than any known liquid. They have been tested in humans and demonstrate a strong safety profile. Current state-of-the-art emulsions give these substances a 1-year shelf-life. A single 100ml bottle carried by a corpsmen has the ability to deliver the equivalent oxygen of 1-2 units of blood (500ml). Furthermore, low volume resuscitation of hemorrhage with high oxygen carrying capacity fluid has the further potential to improve oxygen delivery without impairing hemostasis. The small particle size of a PFOB emulsion, 0.2  $\mu\text{m}$  vs. 8 $\mu\text{m}$  RBC, provides another theoretical advantage that would allow emulsion penetration into compressed and poorly perfused capillaries that cannot be accessed by the larger RBC.

One method to maximize oxygen delivery is to provide oxygen to the patient under hyperbaric pressure. This method directly drives oxygen into solution based on oxygen's solubility for the given solution. In the case of PFOB, a single 100ml injection under 3 atmospheres of pressure, would deliver the equivalent oxygen as 1.5 liters of whole blood. Stated another way, 3 bottles of PFOB under this pressure is adequate to entirely replace the oxygen delivery of blood. Currently the US Navy holds the patent for The Fly-Away Advanced Care System (FAACS). The FAACS is a small 24x15x15 inch hyperbaric chamber that can be carried and inflated to provide critical care access, 3 atmospheres of pressure, and 6 hours of oxygen. Such a system could be used in forward deployment areas and provide critical treatment in combination with PFOB until the soldier arrives to a neurosurgical facility.

Although the use of perfluorochemicals has been used and approved for use in humans in Japan, USA, Europe and Russia, no current approval has been achieved for the second-generation emulsions with long stable shelf-lives. Furthermore no animal or human study has been performed with the combination of hyperbaric and PFOB for ischemic or traumatic injury to the brain. **Thus the goal of this proposal is to test the combined treatment of PFOB emulsion with hyperbaric oxygen in the setting of TBI.**

## Body

The following were the specific tasks that were set out in the original proposal.

### Specific Tasks

- 1) Determine the greatest delay (time) from TBI to onset of treatment that maintains both tissue sparing and functional improvement effects
- 2) Determine the minimal dose of PFOB at 60 minutes after TBI that maintains both tissue sparing and functional improvement effects
- 3) Determine the lowest partial pressure of oxygen at the highest effective dose of PFOB at 60 minutes that maintains both tissue sparing and functional improvement effects.
- 4) Determine the efficacy of PFOB and HBO for tissue sparing and functional improvement in TBI combined with hemorrhage.

### Results

#### **1. Traumatic Brain Injury (TBI) caused reduction of cerebral blood partial pressure oxygen (PBtO<sub>2</sub>) in the lesion.**

Controlled cortical injury (CCI) model causes direct penetration and disruption on the cerebral cortex and sub-cortex structures in experimental animals. The primary insult destroys cerebral blood vessel networks and altered blood flow and subsequently oxygen delivery and utilization. To illustrate the loss of brain tissue partial oxygen pressure (pBtO<sub>2</sub>) in mouse cortex after TBI, we used a fluorescent oxygen sensor (Oxford-Optronix) to quantitatively measure the regional pBtO<sub>2</sub> in both normal and injured brain tissue (1.0 mm deep into cortex).

C57B6 mice (20g body weight, total 11 animals) were anesthetized and a cranial window was opened on the right side of the skull. A controlled cortical impact was delivered onto the right cerebral cortex (6 m/s speed, 1.2 mm deep). The diameter of contusion was 1.5 mm diameter and centered at minus 1.0 mm from Bregma, as shown in Figure 1A. The first pO<sub>2</sub> reading was collected at the intact right cortex before CCI injury. As shown in Figure 1B, the average pO<sub>2</sub> is 21.8 mmHg, which is similar to the published partial oxygen pressure in mice cortex tissue. After CCI injury, the pO<sub>2</sub> at the center of lesion dropped to almost zero (0.21 mmHg), which reflected the disrupted blood flow and oxygen delivery. In the penumbral region, 1mm anterior and posterior from the edge of CCI lesion, the average pO<sub>2</sub> dropped dramatically to average of 3.0 and 3.5 mmHg respectively. At the contra-lateral (un-injured) side of cortex after CCI injury, the average partial oxygen pressure also dropped to 13.3 mmHg. The drop in the uninjured contralateral cortex is consistent with the phenomenon of diaschisis after injury to cerebral cortex. These results demonstrated that disruption of blood vessels network after traumatic brain injury causes hypoxia in the brain tissue surrounding the lesion region.

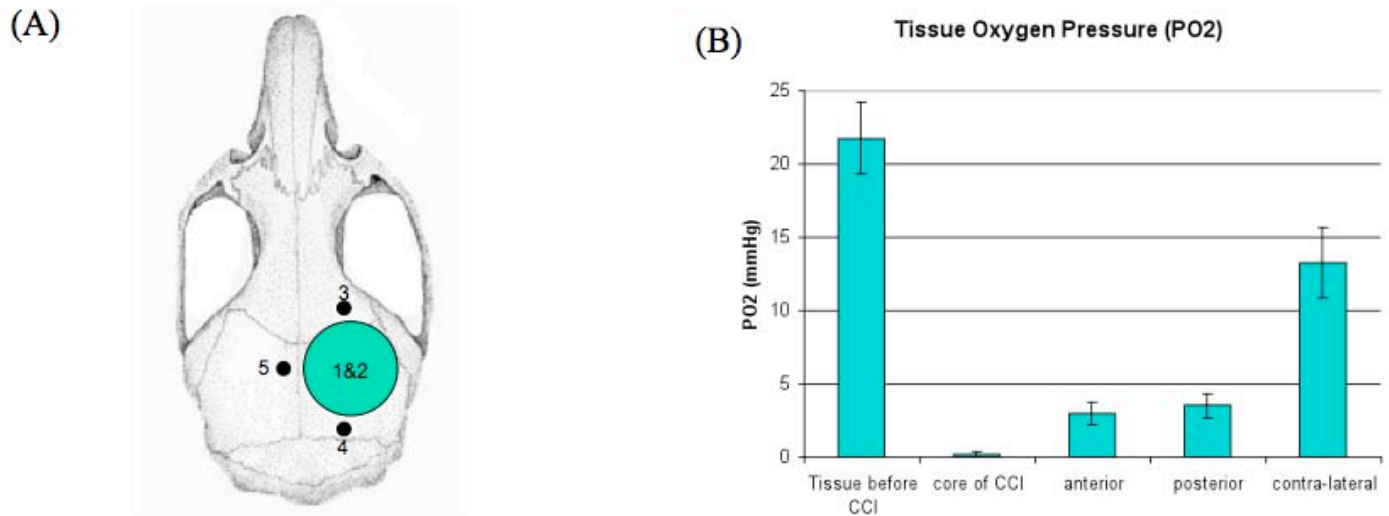


Figure 1. Real-time measurement of oxygen pressure in cortex before and after CCI. (A) The location of cranial window of CCI core (1.5 mm posterior to bregma) and measurement points: 1 and 2, before and after CCI; 3, 1mm anterior to cranial window; 4, 1mm posterior to cranial window; 5, 1mm contra-lateral to cranial window. (B) Tissue partial oxygen pressure (PO<sub>2</sub>) results at different locations before and after CCI. Oxygen pressure unit is mmHg. N = 11, mean  $\pm$  SE.

## 2. Increased Oxygen delivery.

Due to the technical difficulties of performing pBtO<sub>2</sub> measurements within a closed hyperbaric chamber, we performed an experiment at 1 atmosphere of pressure and changed the oxygen delivery to 100% to deliver, or 1 atmosphere absolute (ATA) of oxygen in a stroke model. Fig 2 demonstrates that after 1 hour of occlusion to the middle cerebral artery that increasing the inspired air to 100% (1 ATA) that an increase in cortical pBtO<sub>2</sub> is measured. This increased delivery of oxygen provides a therapeutic protection of brain tissue in a stroke model when delivered at 2 ATA (Fig. 3). Perfluorooctyl bromide (PFOB) has 20 times more capacity to carry oxygen than whole blood. However, our probe cannot measure dissolved oxygen and is only sensitive to partial pressure (gas out of solution) and thus remains unchanged with PFOB treatment. Despite our inability to directly measure the increase in pO<sub>2</sub>, our experiments which supplement up to 16% of total blood volume with PFOB significantly decrease infarct volumes (Table 1).

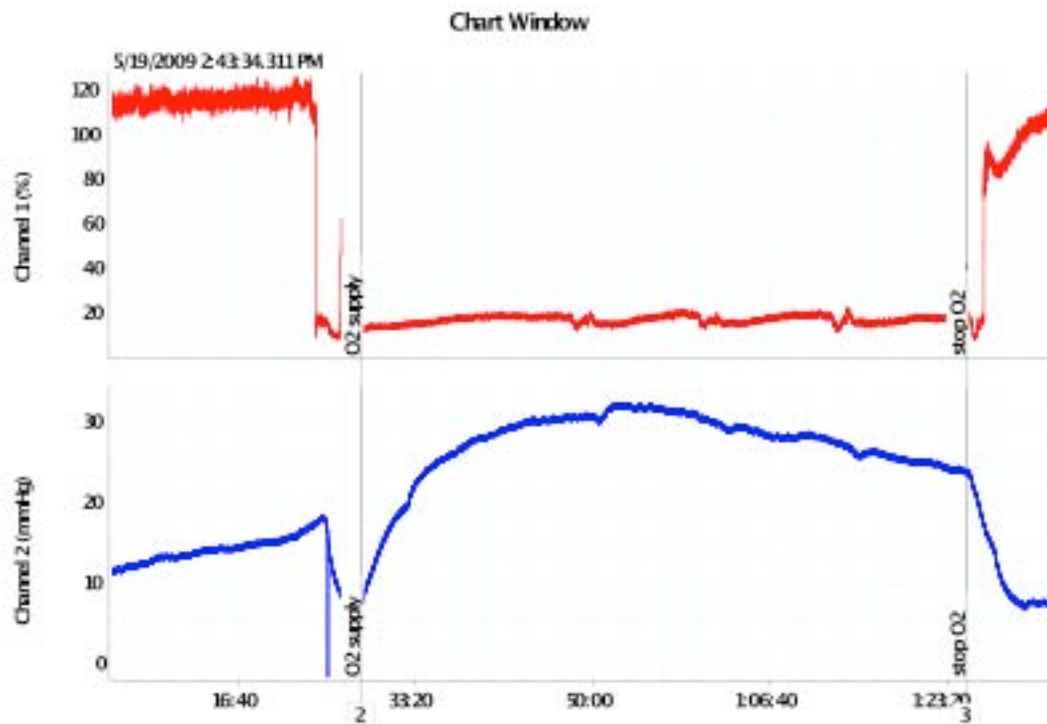


Fig 2. Increase oxygen delivery through ATA manipulation in a stroke model. Both cerebral blood flow (red) and partial pressure of brain tissue oxygen (blue) are measured above. The blood flow dramatically decreases followed by a slower decrease in pBtO<sub>2</sub> when the middle cerebral artery is occluded. The tissue oxygen level increases to supranormal levels once increased oxygen is delivered (O2 supply label) as measured by fluorescent oxygen probe.

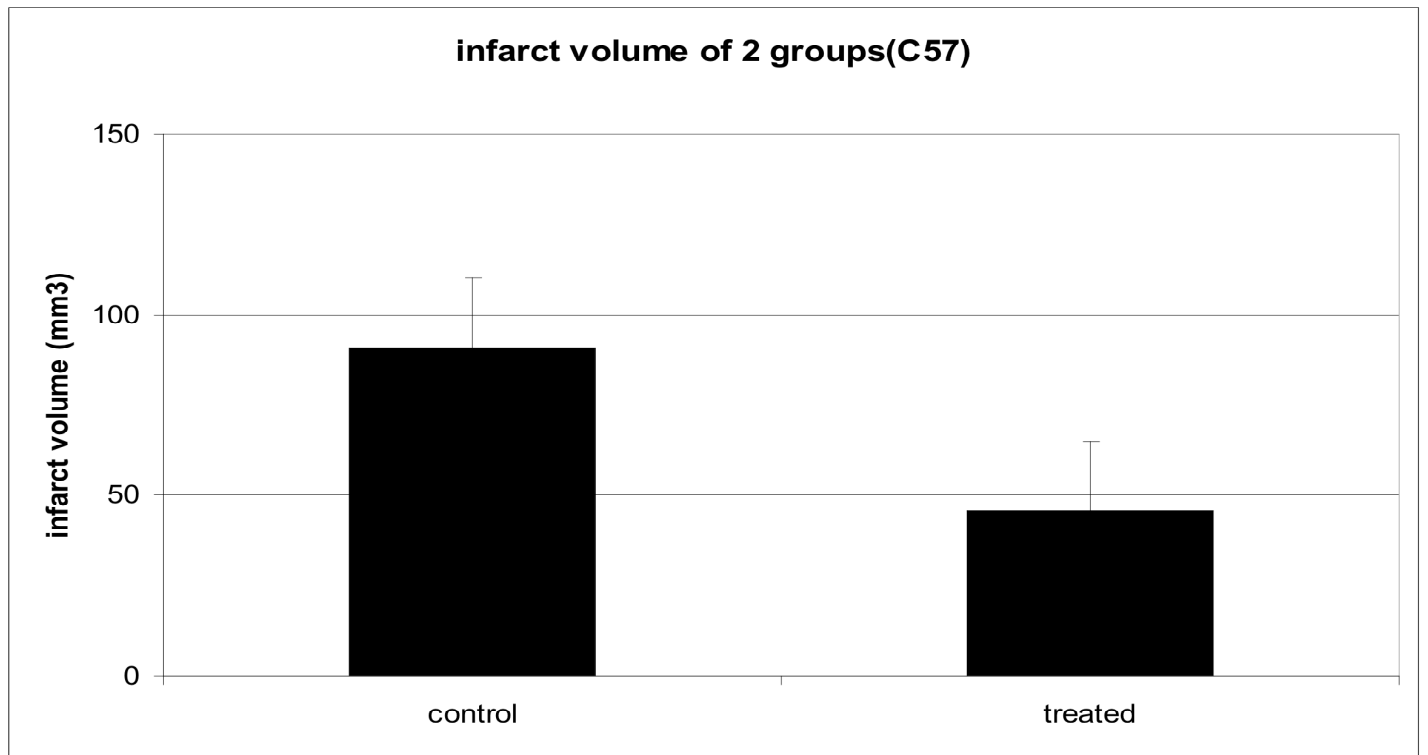


Fig. 3. Increased oxygen saves tissue from infarct. Mice exposed to 2 ATA in a hyperbaric chamber during middle cerebral artery occlusion have significantly less infarct volume than control mice as measured by TTC staining 24 hours after occlusion/reperfusion (N=8, P=0.02).

	4%D+H	8%D+H	12%D+H	16%D+H
N	4	4	8	10
INFARCT VOLUME	65.1±16.2	38.6±7.3	26.3±10.2	22.8±8.0

Table 1. PFOB improves infarct volumes. With increasing drug (D) in addition to 2 ATA hyperbaric treatment (H) there is decreasing infarct volumes as measured by TTC staining.



**3. PFOB, hyperbaric therapy or both have no effect on learning memory for intermediate injury or severe injury groups of CCI.**

Two weeks after the initial CCI injury, all mice were tested in Morris Water Maze to measure the learning memory ability. Invisible, visible and probe tests were performed. In the visible cue and probe tests for 1.2 mm intermediate injury (Fig. 4), showed no significant difference in learning ability between PFOB, PFOB plus hyperbaric chamber, vehicle, and vehicle plus hyperbaric treated group. Furthermore, there were no differences found in the 2.0 mm deep severe injured mice group (Figure 5), no improvement over time. This was probably caused by the deep injury (2.0 mm into cortex) that also destroyed the hippocampus of ipsi-lateral side under the cerebral cortex.

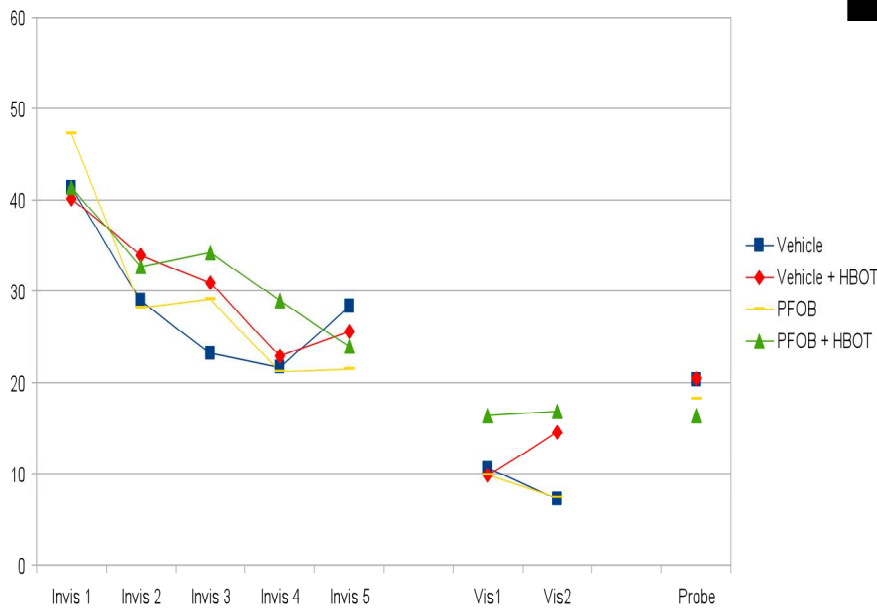


Figure 4. Intermediate injury level 1.2mm CCI, Morris Water Maze. Mice were treated with 16% PFOB 10 minutes after 1.2mm CCI and 1 hour of 2 ATA hyperbaric treatment. There were no significant differences among vehicle, PFOB or Hyperbaric (HBOT) or combination therapy in the invisible trials (Invis), visible (vis) or probe trials.

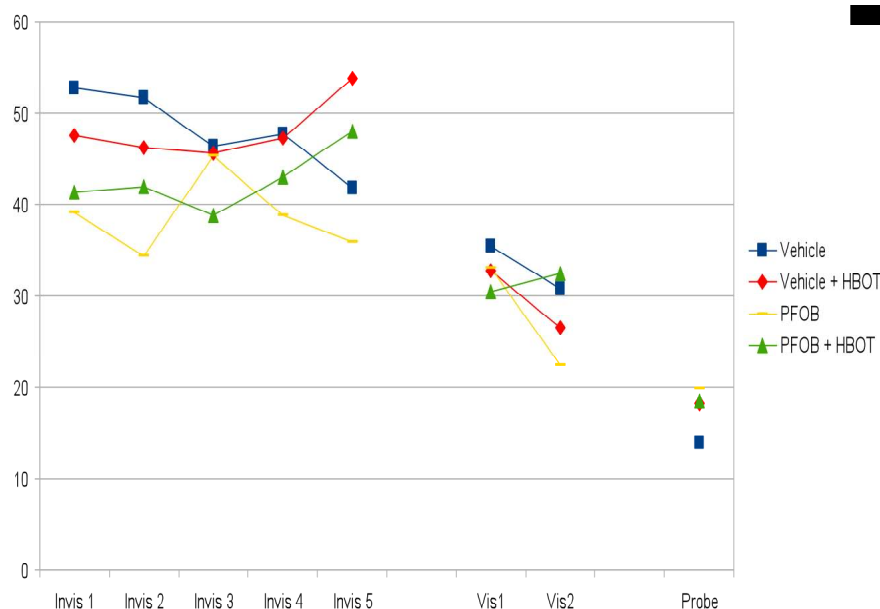


Figure 5. Severe injury level 2mm CCI, Morris Water Maze. Mice were treated with 16% PFOB 10 minutes after 2mm CCI and 1 hour of 2 ATA hyperbaric treatment. There were no significant differences among vehicle, PFOB or Hyperbaric (HBOT) or combination therapy in the invisible trials (Invis), visible (vis) or probe trials, nor was there any demonstration of learning in any group in this severe model.

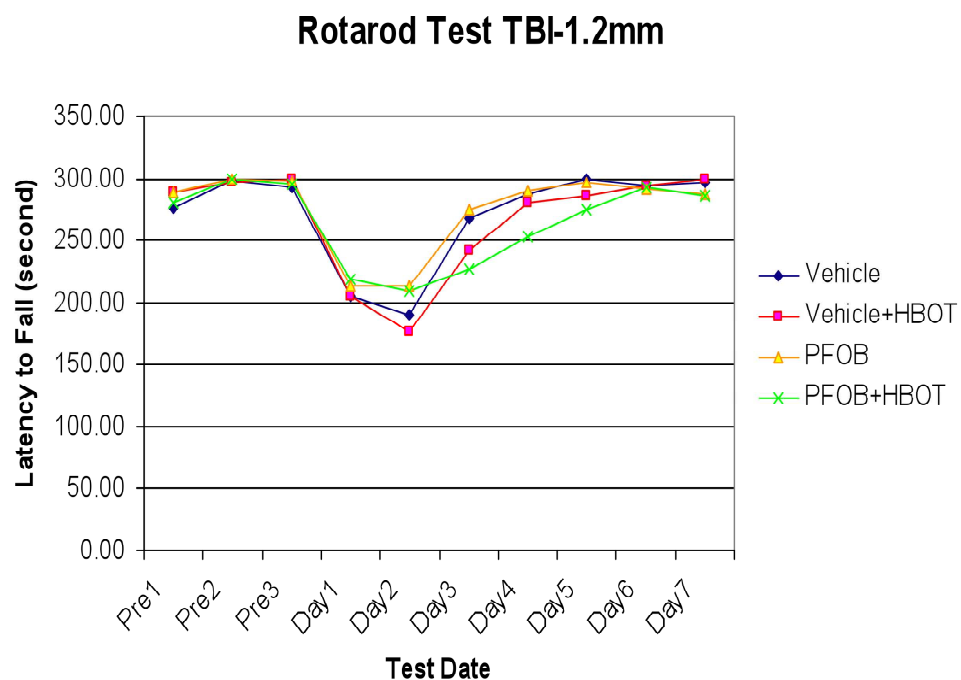
#### 4. PFOB and hyperbaric chamber treatment did not benefit TBI induced Motor function loss and recovery.

The mice were divided into two experimental groups—1.2 mm depth of intermediate and 2.0mm depth of severe CCI injury operated on the Electromagnetic Controlled Cortical Impact Device. This chosen standard was comparable to the lesion volume loss produced in other CCI apparatus (such as the Pneumatic cortical impact device). After CCI injury, the mice were immediately given either a dose of vehicle solution or PFOB solution (16% of total blood volume according to mice's body weight) through jugular vein. The mice were then further divided into two sub-groups to be placed either in regular cages, or inside a hyperbaric chamber supplied with 2ATA of pure oxygen for 1 hour, respectively. Very few animals may display seizure-like activity due to the brain trauma injury. After hyperbaric chamber treatment the mice were also put back in cages for future behavior and histology evaluation.

Motor function recovery test was performed on Rotarod testing device from day 1 to day 7 after CCI injury. As shown in Figure 6, in the mice group that received intermediate (1.2mm deep) CCI injury, the average time of falling out of the Rotarod bar decreased from 300 seconds (pre-injury baseline level) to around 200 seconds during day 1 test. The second day some mice showed worsen performance. Then in the third day most animals started to improve their time staying on the Rotarod bar. By day 5, motor function depicted by Rotarod test had returned to near the pre-injury level for almost all animals. No significant difference of motor function loss and recovery process was observed between PFOB, PFOB plus hyperbaric chamber, vehicle, vehicle plus chamber treated groups.

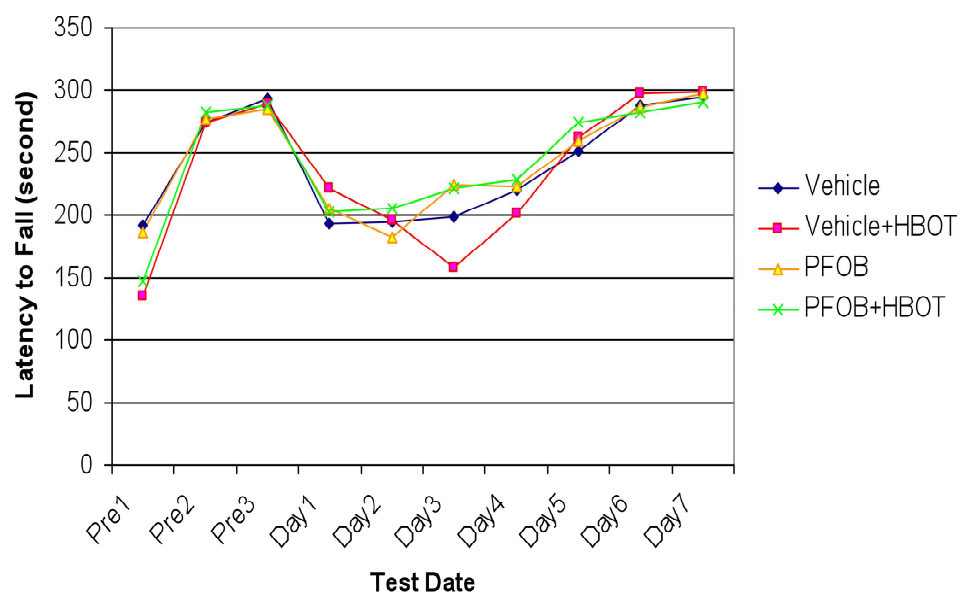
In the mice groups that received more severe injury (2.0 mm deep, Figure 7), similar level of impairment of motor function was also observed in the first day of injury (average around 200 seconds). The recovery rate was somewhat slower compared to the 1.2mm injury group. Only by day six the motor function was recovered to near pre-injury level compared to by day 5 in the 1.2 mm group. Again no significant difference of function loss and recovery rate was found between different vehicle and PFOB treated groups.

Wire Grip, another motor function test was also used to exam the 2.0 mm depth mice group. Again no obvious difference of motor function recovery exists between PFOB and vehicle treated group within one week of testing period. (Figure 8)



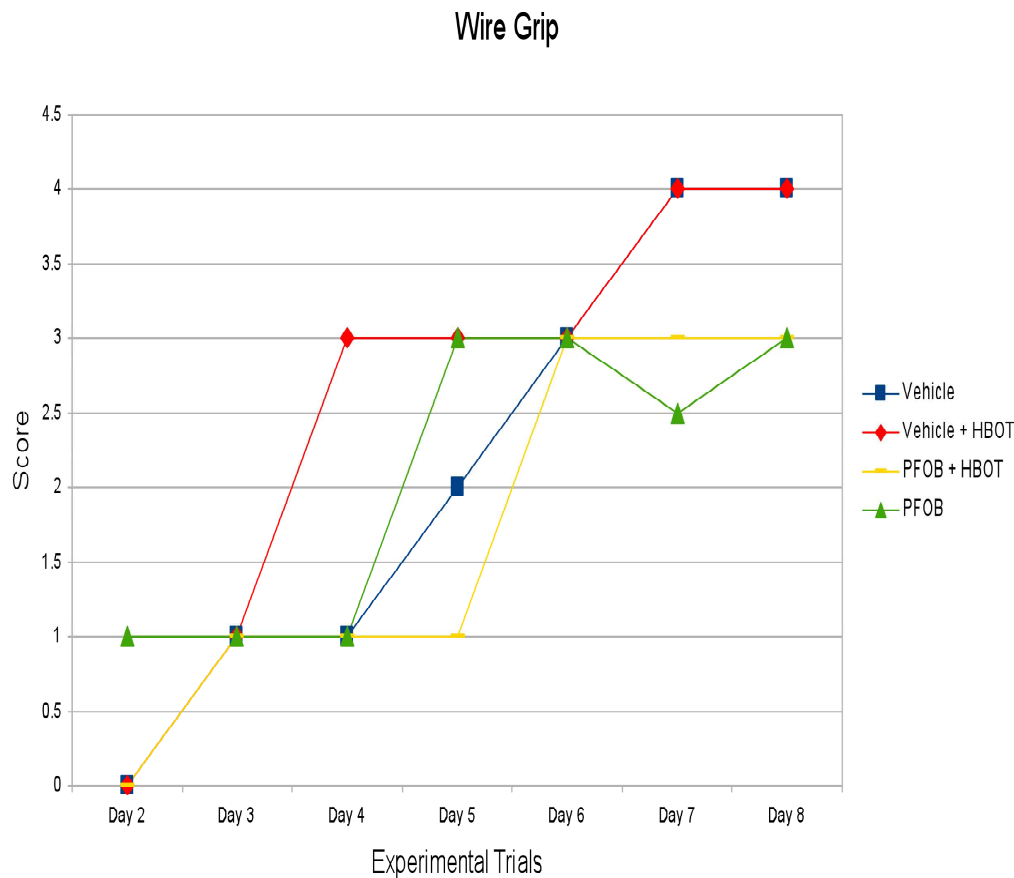
**Figure 6**

## Rotarod Test TBI-2.0mm



**Figure 7**

Figure 6 and 7. Rotarod test scores for mice of mild (1.2mm) and medium (2.0 mm) level TBI. Mice were treated with vehicle, vehicle plus HBOT, PFOB, PFOB plus HBOT 10 minutes after CCI. The scores were count as the latency (second) to fall off rod. Acceleration rate was 0 to 40 rpm in 300 seconds. Scores were counted in three days of pre-training and 1–7 days after TBI. In mild TBI animal number n=8 for each group, in medium TBI n=7 for all groups except n=8 for PFOB+HBOT.



**Figure 8**

Figure 8. Wire grip test score for medium (2.0 mm) level TBI. Score: 0 - Falls off wire in less than 30 secs. 1 - Hangs on wire for at least 30 secs in any fashion. 2 - Bring all four paws back to midline for 5 secs and hangs on wire for at least 30 secs. 3 - Bring all four paws back to midline and wrap tail around wire for 5 secs and hangs on wire for at least 30 secs. 4 - Move along wire using all four paws and tail for 5 secs and hangs on wire for at least 30 secs. 5 - Successfully traverse the wire climb down the pole using all four paws and tail within the given time limit.

##### **5. Lesion volume loss did not change significantly after PFOB and hyperbaric chamber treatment.**

At the end of three weeks period after CCI injury, all mice were sacrificed and the brains were harvested and processed for lesion volume determination. The sections were stained with H&E and lesion volume was measured. In the 1.2 mm intermediate injury group, the lesion area affected only the cerebral cortex layer while in the 2.0 mm injury group the lesions extended deeply into the hippocampus area (not shown). Lesion volume measurement showed that in the 1.2 mm injury group there were no significant differences in lesion volume among groups. In the 2.0 mm injury group, we also didn't observe significant change of lesion volume among groups. Furthermore, there was no correlation with lesion volume and performance on rotarod or water maze performance (data not shown).

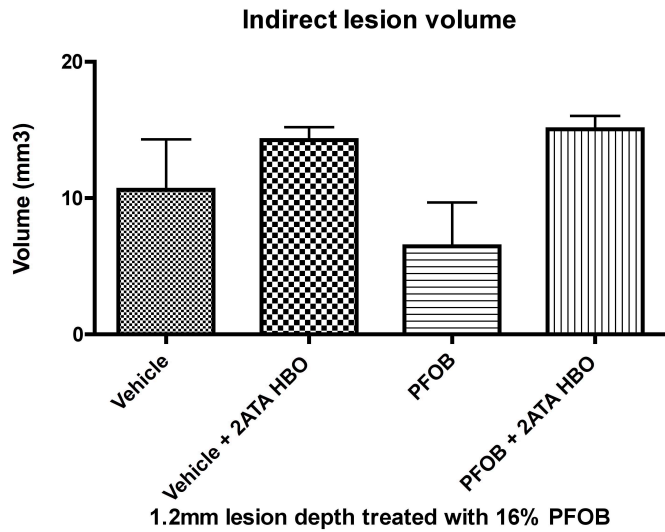


Figure 9A

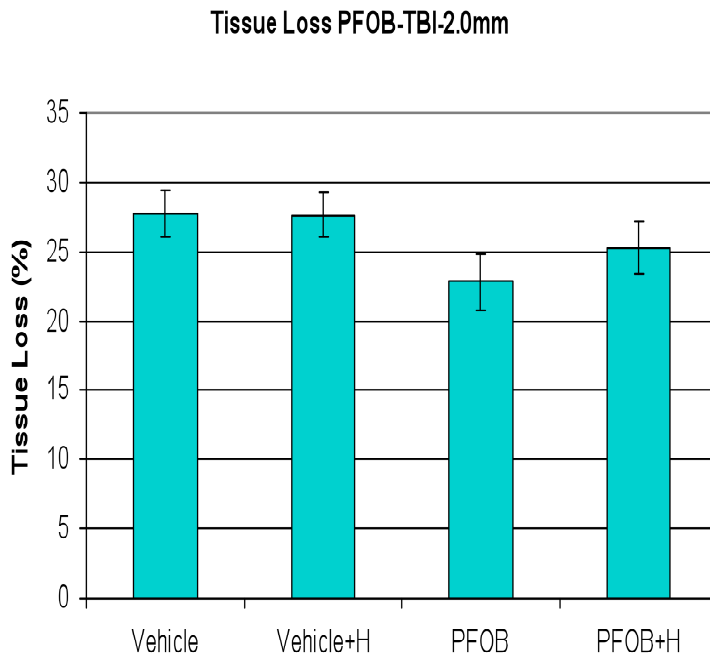


Figure 9B

Figure 9. Brain tissue loss for mice of intermediate (1.2mm) and severe (2.0 mm) level TBI. Freshly frozen brains were sectioned with cryostat to 20  $\mu$ m thickness. The tissue loss was measured stereologically and calculated as the ratio of brain volume of ipsi-lateral to contra-lateral side of TBI (represented as indirect or % volume). (A) Indirect volume loss in 1.2mm lesion depth group shows there is no significant differences ( $n=3-4$ /group). (B) Lesion volume results of 2.0 mm TBI shows no significant difference in lesion volume ( $n= 7-8$ /group). Mean $\pm$ SE.

**6. 3ATA HBO was harmful**

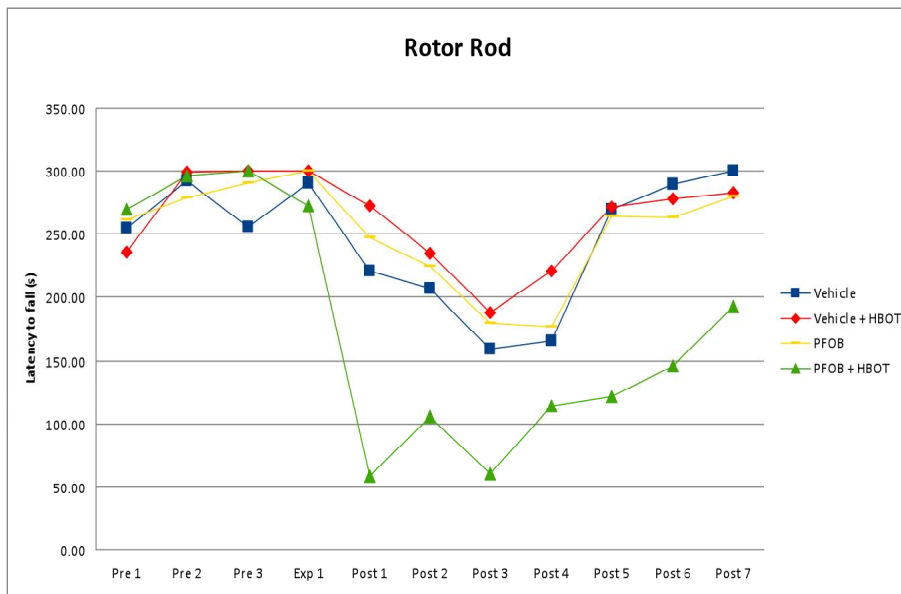
Increasing amount of oxygen exposure by adjusting the ATA up to 3 ATA was not helpful and resulted either in an increased mortality if treated 60 minutes after TBI (Table 2), or if treated earlier (10 minutes after TBI) there was no increased mortality (Table 3) but it resulted in worse functional performance on water maze testing (Figure 10). Therefore this treatment arm of 3ATA with 16% PFOB was eliminated from further testing.

Group	n	mortality
vehicle	3	0
PFOB*	3	2/3(post3,9)
HBO	3	1/3(excluded)
HBO+PFOB*	3	2/3(post4,13)
Sham	4	0

**Table 2 :** 3ATA for 2 hours after a 60-minute delay from TBI, with 16% PFOB demonstrated increased mortality. This treatment arm was discontinued

Group	n	mortality
vehicle	4	1/4 (excluded)
PFOB*	4	0
HBO + vehicle	4	0
HBO + PFOB*	4	0

**Table 3:** 3ATA for 2 hours after a 10-minute delay from TBI, with 16% PFOB had no increased mortality.



**Figure 10:** Worsening performance at 3ATA for 2 hours after a 10-minute delay from TBI, with 16% PFOB despite no increased mortality.

### Key Research Accomplishments

- 1) Demonstrated that PFOB + HBO can improve ischemic disease in stroke model
- 2) Determined mild, intermediate and severe levels of TBI in mouse model with the new electromagnetic CCI machine. Mild = 0.6mm, Intermediate = 1.2mm, Severe = 2.0 mm. These compare to the old pneumatic machine, which had intermediate at 0.6mm and severe at 1.2mm.
- 3) Demonstrated reduced oxygen tension in peritraumatic tissue
- 4) Evaluated a high and low dose of hyperbaric oxygen
- 5) Evaluated a high and intermediate level of trauma
- 6) Evaluated an early and delayed treatment time
- 7) Evaluated a single dose of PFOB, the highest proposed
- 8) Due to lack of efficacy in this CCI TBI model further parameters were not tested and investigations of oxygen delivery after TBI were begun to evaluate NAD/NADP ratios. This work was not completed at time of grant ending.

### Reportable Outcomes

This research resulted in a disseminated abstract USAMRMC. This work resulted in a poster presentation and podium presentation at MRF. This work provided training to 3 individuals. This work provided some background experience for application for NIH funding. This work is in preparation for submission to a peer-reviewed journal.

### Conclusions

PFOB, in combination with HBO, can provide superior tissue salvage than either treatment alone in models of ischemia and reperfusion. However under the current TBI CCI model we demonstrated no consistent tissue salvage or improved behavioral performance under 3ATA or 2ATA of hyperbaric treatment, combined with 16% PFOB, with intermediate or severe levels of trauma at either early or delayed treatment times. Thus, we cannot recommend this avenue for further investigation in a CCI model. This treatment may still provide benefit in a closed head injury model where swelling may encumber tissue perfusion.

**References:** none



## **Appendices: methods section**

### Mice Model of Traumatic Brain Injury (TBI)

Adult male C57 mice (about 20 g, 10 weeks old) were purchased from The Charles River Laboratory, MA. All experiments were performed following institutionally approved protocols in accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals under Massachusetts General Hospital guidelines. Animals were anesthetized and a cranial window (5mm diameter) was opened at right side of the skull. The animals were then underwent mild (1.2mm) and medium (2.0mm) controlled cortical impact (CCI) using a Custom Design & Fabrication Model 6.3 apparatus (speed 6 m/s). The center of the impact was located at minus 1.5 mm from Bregma and 3mm from midline and the diameter is 3 mm. Treated groups received an intravenous infusion of PFOB emulsion at 10 minutes after TBI injury, followed by immediate 1-hour HBO therapy (HBOT) at 2 atmospheres. The dose of PFOB dose was 16% total blood volume. Controls received emulsion vehicle only, vehicle +HBOT, or PFOB only. Animals were returned to cages and kept for future behavior and histology studies.

### Real-time measurement of oxygen pressure in mice cortex

Real-time measurement of brain tissue partial oxygen pressure (1mm deep into cortex) was performed with a device from Oxford Optronix. The PO<sub>2</sub> level was detected at the core of CCI, 1 mm anterior and posterior of CCI cranial window, and 1mm contra-lateral from the CCI window. Oxygen pressure unit is mmHg.

### Motor Function Recovery Test

Rotarod test for motor functional improvement was measured by prior to and from 1 to 7 days after TBI. The scores were count as the latency (second) to fall off rod. Acceleration rate was 0 to 40 rpm in 300 seconds. Scores were counted in three days of pre-training and 1- 7 days after TBI.

Wire grip test was performed in the second week after injury. Score: 0 - Falls off wire in less than 30 secs. 1 - Hangs on wire for at least 30 secs in any fashion. 2 - Bring all four paws back to midline for 5 secs and hangs on wire for at least 30 secs. 3 - Bring all four paws back to midline and wrap tail around wire for 5 secs and hangs on wire for at least 30 secs. 4 - Move along wire using all four paws and tail for 5 secs and hangs on wire for at least 30 secs. 5 - Successfully traverse the wire climb down the pole using all four paws and tail within the given time limit.

### Learning Memory Recovery Test

Morris water maze test for learning memory is performed 14 days after TBI injury, including invisible test, visible test, and probe test. Scores were counted as the average time (second) for mice to reach platform in invisible and visible tests, from four different directions (designated as east, west, north, and south). Probe test was counted as the average time of staying in the designated quarter of the tank.

### Total brain tissue loss measurement after TBI.

Animal brains were harvested at 21 days after TBI. Freshly frozen brains were sectioned with cryostat to 20 µm thickness and then stained with H&E staining. The tissue loss was measured stereologically and calculated as the ratio of brain volume of Ipsi-lateral to contra-lateral side of TBI (%). ANOVA comparisons were made among the 4 groups of volume of tissue loss.